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TITLE:

METHODS AND COMPOSITIONS
FOR INCREASING PROTEIN YIELD
FROM A CELL CULTURE

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METHODS AND COMPOSITIONS FOR INCREASING PROTEIN YIELD FROM A CELL CULTURE

RELATED APPLICATIONS

5 This application claims priority to United States Provisional Application
Ser. No. 60/226,290, filed August 21, 2001, which is incorporated herein by
reference in its entirety.

BACKGROUND OF THE INVENTION

10 Manufacturing of biopharmaceuticals depends on cell cultures that secrete
protein products into surrounding media. Typically, this conditioned media
containing the desired product is used for downstream processing, while a new
batch of fresh media is supplied to the cells. Naturally, increasing cell density in
the bioreactor can make the process more productive. However, the lifespan of
super-dense cultures is much shorter than lower density cultures because
continuous proliferation can reduce available attachment surfaces until cell layers
15 detach from solid support. At this stage, the bioreactor has to be recycled:
washed, sterilized and re-seeded.

20 Cell growth can lead to increased product yield until a maximum is
reached; then the cycle is repeated. During this cycle the period of maximum cell
density and maximum bioreactor efficiency can be relatively short. The length of
this period is determined to a large extent by the proliferation rate of producer
cells. At the beginning of the cycle, when the seeding density is relatively low, it
is advantageous to INCREASE proliferation rate to achieve high cell density
faster. On the other hand, it would be favorable to REDUCE the rate of
proliferation when the maximal density is achieved in order to preserve cell
25 population at its most productive state. Besides increasing the bioreactor cycle at
its production peak, reduced rates of proliferation can channel cell energy from
proliferation to protein production, further increasing yields.

30 Current approaches to increasing the useful time of bioreactor cycle
concentrate on media adjustments at or close to the peak of production. The most
common method is reduction in serum content of the bioreactor media. While

effective in preventing further cell division, this approach can interfere with protein synthesis, thus reducing beneficial effects of decreased cell growth.

Recombinant DNA technology has opened new avenues for the production of useful therapeutic proteins, such as hormones, growth factors, and interferons, in commercial quantities. To economically produce therapeutic proteins at commercial scale, while controlling product quality requires three general steps. First, an effective strategy for maximizing recombinant gene expression, next, a sufficient fermentation process, finally, robust protein recovery and purification processes must be instated.

Elaborate methods of vector construction and cell culture methods are required for production of biopharmaceuticals from mammalian cells. Promoters such as immediate early cytomegalovirus promoter (CMV) can mediate very strong interactions with the transcriptional machinery in most mammalian cellular systems (F. Weber, J. de Villiers, W. Schaffner, *Cell* **36**, 983-92 (1984). Based on this attribute, CMV promoter is frequently used in mammalian expression vectors. High concentrations of protein (mg/ml) are generated using these constructs. The limitation in production of these biopharmaceuticals is generally related to the capacity of the cells to synthesize and secrete the protein product.

Developments in bioprocess engineering of mammalian cells have generally relied on manipulation of culture media components to reduce cell proliferation upon achieving a high density of cells. When the bioreactor is initially seeded with protein-producing cells the efficiency of the process is relatively low because of the low cell density. At this stage cell growth in the bioreactor is the major concern; however, when the cell density reaches its maximum, cell growth becomes detrimental to the system, because cells require additional space and nutrients. Decreasing serum in the media is the most common method of blocking cell proliferation, however, in many cases the core effect of these modifications is reduction of energy level, which is detrimental to the protein synthesis and thus to overall production capacity of the bioreactor.

It would be useful to have a technology that could prevent cell proliferation without affecting protein synthesis to result in increased yields of synthesized

bioproducts. An additional benefit of such technology would be diversion of energy, otherwise spent on reproduction, to sustain/increase protein synthesis.

SUMMARY OF THE INVENTION

5 The invention relates to protein synthesis in general, and increased protein synthetic productivity of cells in particular. By switching the cells from a replicative to a productive state (RP switch), protein biosynthesis can be extended. The productive state is a pseudo-senescent state. This pseudo-senescent state can be induced by transforming the cells with a vector expressing a cell cycle inhibitor. Expression of the cell cycle inhibitor within the cell, because it does not
10 cause cell death, allows for cells to be maintained in culture for longer periods (cell numbers within a culture do not increase and overgrow the growth container). The invention allows for controlled enhanced protein biosynthetic productivity of cell lines for commercial and research purposes.

15 According to one aspect, the invention is a method of increasing yield of a protein from a cell culture, preferably a eukaryotic cell culture, more preferably a mammalian cell culture, by causing a pseudo-senescent state in one or more cells in the cell culture; and collecting a protein fraction from the cell culture.

20 According to another aspect, the invention is a method of increasing yield of a protein from a eukaryotic cell culture, by contacting the cell culture with an expression vector which comprises an inducible transcription regulation element comprising a tetracycline operator element, and collecting a protein fraction from the cell culture.

25 According to yet another aspect, the invention is a transcriptional regulatory element which includes a minimal promoter comprising a TATA sequence, two phased tetracycline operators downstream from the TATA sequence, and two phased tetracycline operators upstream of the TATA sequence.

30 According to still yet another aspect, the invention is an expression vector including a minimal promoter comprising a TATA sequence, two phased tetracycline operators downstream from the TATA sequence, and two phased tetracycline operators upstream of the TATA sequence.

Abbreviations and Definitions

The following abbreviations are used in this disclosure:

CDK, cyclin-dependent kinase;

5 CKI, cyclin-dependent kinase inhibitors;

CMV, cytomegalovirus;

DNA, deoxyribonucleic acid;

env, the retrovirus gene encoding the envelope proteins in the membrane of
the viral particle;

10 gag, the retrovirus gene encoding the core proteins of the viral particle;

G418, geneticin (GIBCO, Inc);

kb, kilobases of nucleic acid;

LTR, long terminal repeat;

MoMLV (or MuLV), Moloney strain murine leukemia virus;

15 mRNA, messenger RNA;

neo, neomycin phosphotransferase;

pA, polyadenylation signal;

pol, the retrovirus gene encoding the viral reverse transcriptase;

RNA, ribonucleic acid;

20 RP, replicative to productive;

PSI, the packaging nucleotide sequence for murine retroviruses;

tTA, Tc-controlled transactivator;

Tet, tetracycline;

TetO, tetracycline operon;

25 TetR, tetracycline repressor;

TFIID, transcription factor IID; and

T-Rex, tetracycline responsive plasmid vector.

30 “cell cycle” means the biochemical process by which mammalian cells
duplicate themselves.

“cyclin-dependent kinase” is a family of enzymes that trigger progression through the cell cycle.

“CDK inhibitors” are proteins produced naturally by cells to block progression through the cell cycle.

5 "defective", means genetically-deficient in nucleotide sequences required to produce infectious viral particles.

"defective retroviral vector", means a retroviral vector containing an incomplete RNA genome capable of infecting a host cell, but incapable of producing a viral infection (i.e., with progeny virus) in that cell which could
10 subsequently infect another cell.

“doxycycline” is a water-soluble tetracycline analog suitable for mammalian cell cultures.

“ecotropic receptor” is a protein expressed on the membrane of rodent cells that contains the binding site for mouse leukemia viral particles.

15 “G418” is a water-soluble form of neomycin suitable for mammalian cell cultures.

"packaged", means assembling the recombinant murine retrovirus genome into an infectious retroviral vector by surrounding the recombinant retroviral RNA with the gag and pol proteins to form a core particle and encapsulating the core
20 particle in a membrane containing the env protein.

"packaging cell", means a cell containing a proviral genome of a first defective retroviral vector that encodes viral proteins sufficient to assemble a second defective retroviral vector into an infectious retroviral vector virion.

“promoter” is a region of DNA where transcription is initiated.

25 "proviral genome", means a defective retroviral vector nucleic acid integrated in the DNA of a host cell.

“pseudo-senescent” cells are cells forced to express a senescent phenotype by manipulation. Pseudo-senescent cells do not include aged cells that have naturally senesced. Cells in a forced or pseudo-senescent state may have
30 prolonged cell culture lifetimes (S. Goldstein, D. P. Singal, *Exp Cell Res* **88**, 359-64 (1974); A. J. Brenner, M. R. Stampfer; and C. M. Aldaz, *Oncogene* **17**, 199-

205 (1998)), are resistant to apoptosis (B. D. Chang, et al., *Proc Natl Acad Sci U S A* **97**, 4291-6 (2000); and D. Javelaud, J. Wietzerbin, O. Delattre, F. Besancon, *Oncogene* **19**, 61-8 (2000) and increase their protein synthetic capacity several fold (B. D. Chang, et al., *Proc Natl Acad Sci U S A* **97**, 4291-6 (2000); V. J. Cristofalo, D. Kritchevsky, *Prog Immunobiol Stand* **3**, 99-105 (1969); and G. H. Stein, L. F. Drullinger, A. Soulard, V. Dulic, *Mol Cell Biol* **19**, 2109-17 (1999).

"retroviral vector" means a genetically-engineered recombinant retrovirus containing a gene of interest, capable of infecting a mammalian cell wherein the gene of interest can become integrated into the genome of the mammalian cell in a manner that promotes the expression of the gene of interest.

"retrovirus", means an infectious RNA virus having an RNA genome that is converted to DNA and integrated into the genome of the host cell.

"selecting", means cloning (e.g., by limiting dilution), killing undesirable cells (e.g., with drugs or toxins), or mechanical (e.g., by fluorescence activated cell sorting) or physical methods (e.g., by microscopic micropipetting) for collecting individual cells with desirable properties.

"sense orientation" means the direction of DNA sequence in a vector to produce a certain peptide or protein.

"tetracycline" is a naturally occurring antibiotic that is active against bacteria by binding to ribosomes and preventing protein synthesis.

"tetracycline repressor" is a helix-turn-helix protein that forms homodimeric complex that binds tightly to the tetracycline operator.

"tetracycline operator" is a 15 bp palindromic sequence (TCCCTATCAGGGAGA; SEQ ID NO:15) that is bound by tetracycline repressor.

"TFIID" is a core protein necessary for transcription of RNA from DNA at a promoter site.

"transactivator" means a protein that binds to regulatory regions of DNA and enhances the expression of its associated gene.

"transcription" means the process by which the sequence of DNA is used as a template to produce a corresponding piece of RNA.

"vector" means an entity of DNA constructed to introduce and express a set of genes in cells.

"viral particles" is used synonymously with "virions" to mean an infectious virus having a ribonucleoprotein core particle surrounded by a membrane containing envelope protein.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. is a diagram of the regulatory elements of the of the LNTcTX with PolyA vector. The regulatory elements include the viral long terminal repeat (LTR) (1), a selection marker (2), and a multiple cloning site (3) flanked by a polyadenylation signal (4) and a regulated promoter (5).

FIG. 2. is a map of the LNTcTX with PolyA vector.

FIG. 3. shows enhanced production of secreted enzyme from cells blocked by RP Shift.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides compositions and methods for producing a pseudo-senescent state in a cell culture for the purpose of increasing protein yield from the culture. By placing the cells in a pseudo-senescent state, the cell density of a culture may be maintained for a longer period without overgrowth of the culture leading to cell death. Essentially, any cell type may be used. Furthermore, the yield of essentially any protein may be improved including secreted proteins, membrane proteins, and intracellular proteins. In preferred embodiments, the protein is secreted by the cells. For example, a hybridoma cell line may be placed in a pseudo-senescent state to increase the yield of monoclonal antibody from a culture of the hybridoma cell culture. In light of the present disclosure, other protein production systems in which the present invention may be applied will become apparent to those of skill in the art.

In preferred embodiments, the invention involves arresting cell division by conditionally expressing known blockers of the cell cycle. The stable introduction of the full-length coding regions of cell cycle inhibitor genes or fragments of such

genes under control of inducible promoters not only stop cell division, but also induce differentiation to a senescence-like (pseudo-senescent) state. Senescence can be characterized by an increase in cell volume, a flattened morphology, and increased protein synthesis. Such cells have longer lifespan and are also substantially more resistant to environmental stresses, such as lowered pH, loss of serum factors, osmotic changes and other impedances that triggers cell death in proliferating population. By this virtue, the present invention increases cell stability and allows higher concentration of secreted products. As a result, complex media or repetitive exchange of media can be circumvented.

In certain embodiments, the present invention allows controlled cell growth arrest without adverse effects on protein synthesis, by increasing the controlled expression of factors that arrest the cell cycle. Such arrest provides two important benefits: (1) it lengthens the phase of maximum production phase of bioreactor cycle; (2) it reduces/eliminates energy requirements for proliferation and thus shifts energy into protein production increasing the output. Combination of both effects can increase overall output from one bioreactor cycle at least ten-fold without any changes in the bioreactor design or principles of operation.

In order to produce a pseudo-senescent state in a cell, embodiments of this invention directly manipulate the progress of the cell cycle. The process of cellular reproduction is known as the cell cycle (A. Murray, T. Hunt, *The Cell Cycle: An Introduction* (Freeman Press, New York, ed. 1st, 1993)). Parental cell goes through a series of steps, eventually producing two daughter cells. Continuous proliferation depends on correct duplication of the genome and of the cell mass and equitable separation of chromosomes and cellular material to the daughter cells.

The cell cycle can be divided into four phases:

S (synthetic) phase: the entire genome is duplicated. In this phase cellular energy is used to synthesize enzymes essential for nucleotide synthesis, followed by DNA replication, proof-reading the finished product and correcting detected mistakes.

M (mitotic) phase: parental cell needs energy to compact the entire duplicated genome into chromosomes, to produce a complex protein scaffold, the mitotic spindle, to attach, align and pull apart the duplicated chromosomes. This is followed by cytokinesis, when the cell divides, forming two identical cells.

5 G1 (gap) phase: the period between M and S phases. The longest phase of the cell cycle. Its length depends on the type of cell as well as its environment. If sufficient nutrition or growth stimulus is not available cells will stop in G1 and not enter S phase (A. Murray, T. Hunt, *The Cell Cycle: An Introduction* (Freeman Press, New York, ed. 1st, 1993)). Following exit from M, cells can escape from
10 the cell cycle into a non dividing state; this is usually followed by terminal differentiation.

G2 phase: the period after S phase and before mitosis. Cells generally do not spend much time in this phase, but divide soon after S phase is complete.

Precise coordination of the cell cycle events is required for successful
15 reproduction. Completion of the S phase is essential before the cell passes its genetic material to daughter cells. Any mistake at this stage can be fatal to the progeny, and cells have developed elaborate mechanisms for controlled entry into S and M phases. The paradigm of cell cycle regulation requires orderly execution of cell cycle events, so that the completion of one event is necessary for the
20 beginning of the next (L. Hartwell, K. Mortimer, J. Culotti, M. Culotti, *Genetics* 74, 267 (1973)). Regulatory feedback controls, that keep the cell cycle from progressing if an essential event does not occur, are called checkpoints.

Genes, responsible for controlled progression of cell cycle, were first described as cell division cycle (cdc) genes in budding yeast (L. H. Hartwell, J. Culotti, J. R.
25 Pringle, B. J. Reid, *Science* 183, 46-51 (1974)); (L. H. Hartwell, *Bacteriol Rev* 38, 164-98 (1974)). The majority of cdc genes turned out to be either protein kinases or protein phosphatases; one of the most important among them is protein kinase cdc2. Homologues to cdc2, called cyclin-dependent kinases (CDKs) regulate the cell cycle in higher eukaryotes. CDK-mediated transfer of phosphate groups to
30 target proteins facilitates their activation or repression, which ultimately results in the progress of the cell cycle. Activation of CDKs depends on their association

with protein co-factors - cyclins (D. J. Lew, V. Dulic, S. I. Reed, *Cell* **66**, 1197-206 (1991)).

Each phase of the cell cycle is characterized by a unique pattern of CDK activity (C. J. Sherr, *Cell* **73**, 1059-65 (1993); C. J. Sherr, *Cell* **79**, 551-5 (1994); and S. van den Heuvel, E. Harlow, *Science* **262**, 2050-4 (1993)). Eight CDKs have been identified in mammalian cells, and most are active in certain phases of the cell cycle. Thus, progression through G1 depends on the activities of CDK2, CDK3, CDK4, and CDK6, while CDK2 and cdc2 are active in S phase, and cdc2 governs entry and exit into mitosis.

Likewise, cyclins are a group of related proteins that contain a conserved region of homology (the cyclin box) and are expressed in specific phases of the cell cycle (D. O. Morgan, *Nature* **374**, 131-4 (1995)). Cyclin levels are rate limiting for the actions of CDKs.

D-type cyclins and cyclin E are expressed in G1 phase of the cycle, when they are involved in regulation of entry into S phase (Koff, et al., *Cell* **66**, 1217-28 (1991); Y. Xiong, H. Zhang, D. Beach, *Cell* **71**, 505-14 (1992); and C. J. Sherr, J. Kato, D. E. Quelle, M. Matsuoka, M. F. Roussel, *Cold Spring Harb Symp Quant Biol* **59**, 11-9 (1994)). These cyclins associate with CDK4/6 and CDK2, respectively. There are three types of D cyclins (F. Weber, J. de Villiers, W. Schaffner, *Cell* **36**, 983-92 (1984); A. Murray, T. Hunt, *The Cell Cycle: An Introduction* (Freeman Press, New York, ed. 1st, 1993); and L. Hartwell, K. Mortimer, J. Culotti, M. Culotti, *Genetics* **74**, 267 (1973)), and these are expressed in a cell-type specific fashion (C. J. Sherr, *Cell* **73**, 1059-65 (1993); C. J. Sherr, *Cell* **79**, 551-5 (1994)). Cyclin D is expressed early in G1 when quiescent (non-dividing) cells are stimulated by environmental or growth factors to enter the cell cycle. Cyclin D levels remain high as long as mitogen levels are elevated. These proteins are labile (half-life of 20 minutes), and their production depends on the continued presence of mitogens (C. J. Sherr, J. Kato, D. E. Quelle, M. Matsuoka, M. F. Roussel, *Cold Spring Harb Symp Quant Biol* **59**, 11-9 (1994)). Hence, growth factor or serum deprivation lowers cyclin D levels and halts the cell cycle in G1. Forcing the expression of cyclin D accelerates progression through G1 (D.

E. Quelle, et al., *Genes Dev* **7**, 1559-71 (1993); and D. Resnitzky, M. Gossen, H. Bujard, S. I. Reed, *Mol Cell Biol* **14**, 1669-79 (1994)) and its inhibition blocks cells in G1 (V. Baldin, J. Lukas, M. J. Marcote, M. Pagano, G. Draetta, *Genes Dev* **7**, 812-21 (1993)). Cyclin E activity is also required in G1, although following cyclin D activity (M. Ohtsubo, A. M. Theodoras, J. Schumacher, J. M. Roberts, M. Pagano, *Mol Cell Biol* **15**, 2612-24 (1995); J. A. Knoblich, et al., *Cell* **77**, 107-20 (1994)). Cyclin E activity peaks at the G1-S boundary, and decays as S phase progresses (M. Ohtsubo, A. M. Theodoras, J. Schumacher, J. M. Roberts, M. Pagano, *Mol Cell Biol* **15**, 2612-24 (1995); A. Koff, et al., *Science* **257**, 1689-94 (1992); and V. Dulic, E. Lees, S. I. Reed, *Science* **257**, 1958-61 (1992)). Cyclin E is regulated transcriptionally by E2F and also by proteolysis, and is required for entry into S phase (D. Resnitzky, M. Gossen, H. Bujard, S. I. Reed, *Mol Cell Biol* **14**, 1669-79 (1994); M. Ohtsubo, A. M. Theodoras, J. Schumacher, J. M. Roberts, M. Pagano, *Mol Cell Biol* **15**, 2612-24 (1995); J. A. Knoblich, et al., *Cell* **77**, 107-20 (1994); and M. Ohtsubo, J. M. Roberts, *Science* **259**, 1908-12 (1993)).

Later cell cycle transitions are mediated by cyclin A and B. Cyclin A associates with CDK2 and cdc2 and its activity is required in S phase and for the G2-M transition (M. Pagano, R. Pepperkok, F. Verde, W. Ansorge, G. Draetta, *Embo J* **11**, 961-71 (1992); A. Giordano, et al., *Cell* **58**, 981-90 (1989); and F. Girard, U. Strausfeld, A. Fernandez, N. J. Lamb, *Cell* **67**, 1169-79 (1991)). Cyclin B associates with cdc2 and regulates mitotic entry and exit.

Cyclin-dependent kinases (CDKs) are also regulated by phosphorylation and dephosphorylation (D. O. Morgan, *Nature* **374**, 131-4 (1995)). The site of CDK activation is a conserved threonine residue within a T loop (H. L. De Bondt, et al., *Nature* **363**, 595-602 (1993)). The binding of cyclin and the phosphorylation of the CDK move the T loop away from the catalytic site of the enzyme allowing substrate to bind. Conversely, phosphorylation of tyrosine in the N-terminal region inhibits CDK activity. Enzymes that inactivate CDKs by adding phosphate to these tyrosine groups are conserved in many species and are known as weel and mik1 kinases (R. Heald, M. McLoughlin, F. McKeon, *Cell* **74**, 463-74 (1993); M. Igarashi, A. Nagata, S. Jinno, K. Suto, H. Okayama, *Nature* **353**, 80-3 (1991); K.

Lundgren, et al., *Cell* **64**, 1111-22 (1991); and P. Russell, P. Nurse, *Cell* **49**, 559-67 (1987)). Dephosphorylation of CDKs is mediated by cdc25 phosphatase. A balance of these activities sets a threshold for CDK activation and determines mitotic entry. Thus, CDKs, through their ability to regulate cyclins, play a central role in controlling cell proliferation.

A critical regulator of cell cycle progression in mammalian cells is the family of Rb proteins. Rb proteins undergo phosphorylation during G1, which modifies its interaction with a critical transcription factor E2F (K. Buchkovich, L. A. Duffy, E. Harlow, *Cell* **58**, 1097-105 (1989); S. Mittnacht, et al., *Embo J* **13**, 118-27 (1994); and J. R. Nevins, *Science* **258**, 424-9 (1992)). E2F transcription factors are heterodimeric DNA binding proteins composed of one E2F factor and one DP factor that are required for the transcriptional regulation of many proteins needed for S phase progression (W. G. Kaelin, Jr., et al., *Cell* **70**, 351-64 (1992); K. Helin, et al., *Cell* **70**, 337-50 (1992); C. L. Wu, L. R. Zukerberg, C. Ngwu, E. Harlow, J. A. Lees, *Mol Cell Biol* **15**, 2536-46 (1995)). There are five known E2F factors, and 3 DP factors. When complexed with Rb, E2F is inactive or may even function as a repressor, thereby silencing E2F-dependent promoters, which, in turn, arrests the cell cycle for a lack of the needed gene products. Phosphorylation of Rb regulates E2F activity. Unphosphorylated Rb avidly binds E2F in early G1, but its phosphorylation at multiple sites lowers its affinity for E2F and releases it to complex with DP in late G1.

The CDKs are responsible for the phosphorylation of Rb. The Rb protein has eight consensus phosphorylation sites and CDKs complexed with cyclin D, E, and A have Rb kinase activities (J. A. Lees, K. J. Buchkovich, D. R. Marshak, C. W. Anderson, E. Harlow, *Embo J* **10**, 4279-90 (1991); S. F. Dowdy, et al., *Cell* **73**, 499-511 (1993); and M. E. Ewen, et al., *Cell* **73**, 487-97 (1993)). Cyclin D-CDK 4 has very high affinity for dephosphorylated Rb (J. Kato, H. Matsushime, S. W. Hiebert, M. E. Ewen, C. J. Sherr, *Genes Dev* **7**, 331-42 (1993)). Cyclin B-CDK4/6 and cyclin E-CDK2 cooperate to inactivate the E2F binding of Rb. The cyclin D function is not essential in Rb-deficient cell lines, suggesting the function of D-type cyclins of promoting G1 phase progression (S. Bates, et al., *Oncogene* **9**,

1633-40 (1994); and J. Lukas, et al., *Nature* **375**, 503-6 (1995)). Cyclin E-CDK2 may also be necessary for G1/S phase transition (M. Ohtsubo, A. M. Theodoras, J. Schumacher, J. M. Roberts, M. Pagano, *Mol Cell Biol* **15**, 2612-24 (1995)).

Thus, progression of the cell cycle involves many factors within the cell.

In light of the present disclosure, one of skill in the art would recognize that one or several of these factors may be utilized or targeted to cause a pseudo-senescent state in a cell. Described below are examples of such.

CDK INHIBITORS (CKIs)

An important aspect of the present invention is the inhibition of cell proliferation. Because CDKs play an essential role in cell proliferation, inhibitors of CDK activity are particularly useful in the compositions and methods of the present invention. All organisms express proteins that directly bind to and inhibit CDK activity (C. J. Sherr, J. M. Roberts, *Genes Dev* **9**, 1149-63 (1995); and M. Peter, I. Herskowitz, *Cell* **79**, 181-4 (1994)). These inhibitors provide another means of cell cycle control in response to diverse stimuli.

Mammalian cells express two classes of CKIs that are distinguished by their CDK targets. The members of the Cip/Kip family of CKIs are universal inhibitors, and INK4 proteins are specific for CDK4/6 inhibition (C. J. Sherr, J. M. Roberts, *Genes Dev* **9**, 1149-63 (1995)). The Cip/Kip family members are p21, p27, and p57. Over-expression of these gene products blocks cells in G1 phase in culture. They are able to inhibit all cyclin-CDK complexes in vitro. These proteins bind avidly to cyclin-CDK complexes, more so than to the factors separately.

p21 was first identified as a component of cyclin-CDK complexes in proliferating cells and as a protein induced as cells become senescent (Y. Xiong, H. Zhang, D. Beach, *Genes Dev* **7**, 1572-83 (1993); and A. Noda, Y. Ning, S. F. Venable, O. M. Pereira-Smith, J. R. Smith, *Exp Cell Res* **211**, 90-8 (1994)). p21 has two functional domains, an N-terminal CDK binding region, and a carboxy-terminal region that associates with PCNA, a processing factor for DNA polymerase delta (S. Waga, G. J. Hannon, D. Beach, B. Stillman, *Nature* **369**, 574-8 (1994); H. Flores-Rozas, et al., *Proc Natl Acad Sci U S A* **91**, 8655-9 (1994); and

Y. Luo, J. Hurwitz, J. Massague, *Nature* **375**, 159-61 (1995)). One role proposed for p21 is to mediate cell cycle arrest.

The CDK inhibitor p27 is structurally related to p21 (K. Polyak, et al., *Cell* **78**, 59-66 (1994); and H. Toyoshima, T. Hunter, *Cell* **78**, 67-74 (1994)). p21 and p27 share significant N-terminal homology within the CDK inhibitory domain. p27 does not contain a PCNA interaction domain. Unlike p21, p27 is not regulated by p53, p27 levels do respond to a number of mitogenic or anti-mitogenic stimuli (C. J. Sherr, J. M. Roberts, *Genes Dev* **9**, 1149-63 (1995)). In general, p27 levels are low in dividing cells, and elevated in growth-arrested cells. The regulation of p27 is complex, involving mechanisms at the transcriptional, translational and posttranslational levels (L. Hengst, S. I. Reed, *Science* **271**, 1861-4 (1996)).

The mechanism of p27 inhibition has been clarified by crystal structure of p27 bound to the cyclin A-CDK2 complex (A. A. Russo, P. D. Jeffrey, N. P. Pavletich, *Nat Struct Biol* **3**, 696-700 (1996)). Separate domains of p27 interact with cyclin A and CDK2. Dramatic alterations in the structure of CDK2 upon p27 binding implicate a distortion of the ATP binding site resulting in the inhibition of CDK2 kinase activity.

Less is known about p57, which was cloned by its homology to p27 (M. H. Lee, I. Reynisdottir, J. Massague, *Genes Dev* **9**, 639-49 (1995); S. Matsuoka, et al., *Genes Dev* **9**, 650-62 (1995)). Both amino and carboxy-terminal inhibitory domains are similar to p27, however, its expression is restricted to terminally differentiated tissues.

The INK4 family of CKIs includes four structural proteins (p15, p16, p18, and p19), each of which contains four ankyrin repeats (C. J. Sherr, J. M. Roberts, *Genes Dev* **9**, 1149-63 (1995)). The first member of this family to be identified, p16, was found to be associated with CDK4 in transformed cells and subsequently was identified as a tumor suppressor in familial melanoma (M. Serrano, G. J. Hannon, D. Beach, *Nature* **366**, 704-7 (1993); R. J. Sheaff, J. M. Roberts, *Curr Biol* **5**, 28-31 (1995); and A. Kamb, *Cold Spring Harb Symp Quant Biol* **59**, 39-47 (1994)). INK4 proteins bind to monomeric CDK4/6 subunits, preventing their

association with D-type cyclins, and INK4 proteins also can inhibit the activity of cyclin D-CDK4/6 complexes.

The INK4 proteins p15, p18, and p19 are expressed ubiquitously in mouse tissues and cultured cells, and the expression of p19 oscillates within the cell cycle (D. E. Quelle, et al., *Oncogene* **11**, 635-45 (1995); and H. Hirai, M. F. Roussel, J. Y. Kato, R. A. Ashmun, C. J. Sherr, *Mol Cell Biol* **15**, 2672-81 (1995)). Although p15 is involved in the anti-proliferative actions of TGF- β , the physiological roles of INK4 remain unknown. The frequent deletions of p15 and p16 in primary tumors and the high incidence of tumors in p16-deficient mice indicate that these proteins play a critical role in maintaining normal growth control (M. Serrano, et al., *Cell* **85**, 27-37 (1996)).

Several independent laboratories have demonstrated successful arrest of asynchronous cell culture in G1 phase of the cell cycle upon introduction (A. Noda, Y. Ning, S. F. Venable, O. M. Pereira-Smith, J. R. Smith, *Exp Cell Res* **211**, 90-8 (1994); J. Vlach, S. Hennecke, K. Alevizopoulos, D. Conti, B. Amati, *Embo J* **15**, 6595-604 (1996); and T. K. Kwon, A. A. Nordin, *Biochem Biophys Res Commun* **238**, 534-8 (1997)) or upon induction (B. B. McConnell, M. Starborg, S. Brookes, G. Peters, *Curr Biol* **8**, 351-4 (1998); L. Fang, et al., *Oncogene* **18**, 2789-97 (1999); L. Uhrbom, M. Nister, B. Westermarck, *Oncogene* **15**, 505-14 (1997); and B. D. Chang, et al., *Proc Natl Acad Sci U S A* **97**, 4291-6 (2000)) of specific inhibitors of cyclin-dependent kinases (CKIs). Accumulated evidence indicate that expression of p16, p27 or p14ARF arrest cells in G1 phase of the cell cycle (J. Vlach, S. Hennecke, K. Alevizopoulos, D. Conti, B. Amati, *Embo J* **15**, 6595-604 (1996); L. Uhrbom, M. Nister, B. Westermarck, *Oncogene* **15**, 505-14 (1997); and G. P. Dimri, K. Itahana, M. Acosta, J. Campisi, *Mol Cell Biol* **20**, 273-85 (2000)); apparently, in many instances p21 expression will also have the same effect (A. Noda, Y. Ning, S. F. Venable, O. M. Pereira-Smith, J. R. Smith, *Exp Cell Res* **211**, 90-8 (1994); and L. Fang, et al., *Oncogene* **18**, 2789-97 (1999)), although prolonged expression of this CKI may initiate apoptosis (Y. P. Tsao, et al., *J Virol* **73**, 4983-90 (1999)).

In certain embodiments of the present invention, CKI expression may be stimulated within a cell or be the result of expression of a heterologous copy of the CKI introduced into the cell. In preferred embodiments, more than one different heterologous CKI is introduced into the cell. For example, heterologous copies of p16, p21, and p57 or p27 may be introduced into a cell.

When a heterologous copy of a CKI is introduced into a cell, it is preferable that the CKI gene is encoded within an expression vector. Even more preferable is that the expression vector be maintained in the cell during cell division. Thus, a retroviral vector, because it is replicated and maintained within genome of the host cell, is particularly useful. Furthermore, because even low levels of expression of the CKI may prevent cell division, it is preferable that expression of the CKI be tightly regulated. Expression of the CKI is turned off to allow growth of the cells within a culture. When the cells reach a desired concentration, the CKI may be turned on, halting cell growth by the production of a pseudo-senescent state.

Of course, other compounds and compositions may be used to produce a pseudo-senescent state within cells. In addition to CKIs, other genes may be used or targeted, such as, E2F2, E2F4 and the cyclins D1 and D2. Current data indicate that E2F family members can act either as activators or as sequesterors of Rb family members. Since E2F2 most frequently acts as an activator (DeGregori *et al.*, 1997), blocking its expression using inducible antisense constructs achieves cell cycle arrest. E2F4, on the other hand, has been implicated in sequestering pRb/p130 (Fabrizio *et al.*, 1999; Furukawa *et al.*, 1999), acting as a negative regulator of the cell cycle. Thus, to arrest cells in G1, an inducible sense-oriented construct of E2F4 is used. Apparently, similar relationship exists between cyclins D1 and D2: D1 is an immediate early gene, induced after serum stimulation of resting fibroblasts (Won *et al.*, 1992). To arrest cells in G1 an antisense construct of cyclin D1 is to be used. Cyclin D2, on the other hand, is induced in senescent cells (Meyyappan *et al.*, 1998); it is likely that it counteracts activity of D1, possibly by sequestering some common factors. This gene may be used as a sense-oriented construct to induce pseudo-senescence.

To further increase cell endurance and to optimize the pseudo-senescent phenotype, additional modifications of producer cells may be required; depending on the cell type these may include introduction of wild type p53, E2F4, cyclin D2, JunB, NF- κ B, HSP72. When multiple expression vectors are introduced it is preferable to express a different resistance marker in order to facilitate selection of appropriate expressing clones.

Other means of blocking cell cycle progression use small molecules or peptides. The small molecules or peptides may be kinase inhibitors. Examples includes compounds that inhibit the kinase activities of cyclin-CDKs, such as the paullones, which selectively inhibit CDK1/cyclin B (Kunick, 1999; Link *et al.*, 1999; Schultz *et al.*, 1999), butyrolactones, that inhibit CDKs 1,2 and 5 (Furukawa *et al.*, 1996; Kitagawa *et al.*, 1994; Kitagawa *et al.*, 1993; Suzuki *et al.*, 1999) competitors of the STP-binding domain, such as olomoucine, purvalanol A, and roscovitine (Abraham *et al.*, 1995; De Azevedo *et al.*, 1997; Gray *et al.*, 1998; Meijer *et al.*, 1997). Addition of these compounds in the growth media promotes pseudo-senescence and may be used to further enhance production of proteins.

Other examples include protease inhibitors. CDK inhibitors, such as p21, p27, are normally turned over at very high rates. These proteins have half-lives of about 20 min in cell culture (Baldin *et al.*, 1993; Quelle *et al.*, 1993). Blocking the turnover of CKIs using a proteasome inhibitor, for example MG132 (Hunt *et al.*, 1999), will sustain high levels of CKIs thereby enhancing the cell cycle arrest produced by the RP shift.

Other compound may be used. Flavopiridol blocks CDK activity by interfering with cyclin D binding to CDK2 (Carlson *et al.*, 1996; Patel *et al.*, 1998). However, flavopiridol causes enhanced apoptosis at the concentrations used in these studies (Schwartz *et al.*, 1997). Thus, it is preferred that, if flavopiridol is used, it is used at concentrations that do not cause apoptosis. Likewise, 7-hydroxystaurosporine (UCN01), which was initially developed as a selective protein kinase C inhibitor, has an anti-tumor effect on several human cancer cell lines in vivo (Kawakami *et al.*, 1996). UCN01 inhibits the kinase

activity of CDKs and promotes accumulation of dephosphorylated Rb thus halting cells in G1 (Akiyama *et al.*, 1997; Gong *et al.*, 1994; Schnier *et al.*, 1994). This compound also produces apoptosis at higher concentrations (Shao *et al.*, 1997). It is likely that low concentrations of this agent could augment the RP shift.

5 Peptides that may be used include Cy region peptides. The cyclin binding Cy motif of the CIP/KIP family of CDK inhibitors (Chen *et al.*, 1996) can interact with the cyclins independently of CDK2. The cyclin-binding motifs of p21 are required for the optimal inhibition of cyclin-CDK kinases in vitro and for growth suppression in vivo. Peptides containing only the N-terminal or C-terminal motif
10 of p21 partially inhibit cyclin-CDK kinase activity in vitro and DNA replication in *Xenopus* egg extracts. A Cy motif is found near the N terminus of Cdc25A that is separate from the catalytic domain (Saha *et al.*, 1997). Mutations in this motif disrupt the association of Cdc25A with cyclin E- or cyclin A-CDK2 in vitro and in vivo and selectively interfere with the dephosphorylation of cyclin E-CDK2. A
15 peptide based on the Cy motif of p21 competitively disrupts the association of Cdc25A with cyclin-CDKs and inhibits dephosphorylation of the kinase. p21 inhibits Cdc25A-cyclin-CDK2 association and dephosphorylation of CDK2. Conversely, Cdc25A associates with cyclin-CDK and protects it from inhibition by p21. Cdc25A also protects DNA replication in *Xenopus* egg extracts from
20 inhibition by p21. Thus, cdc25A and p21 compete for binding with cyclin-CDK complexes. The association of cdc25A, p21, cyclins and CDKs is mediated, in part, by the Cy motif. The Cy motif sequence is found in many proteins involved in cell cycle dynamics (See Table 1).

Table 1. Sequences of Cy motif in Cell Cycle Related Proteins

Protein with Cy motif	Motif Amino Acid Sequence
E2F1	KRRLDL (SEQ ID NO: 3)
E2F2	KRKLDL (SEQ ID NO: 4)
E2F3	KRRLEL (SEQ ID NO: 5)
P107Rb	KRRLFG (SEQ ID NO: 6)
P130Rb	KRRLFV (SEQ ID NO: 7)
Cdc6	GRRLVF (SEQ ID NO: 8)
Myt1	PRNLLS (SEQ ID NO: 9)
Cdc25a	RRRLLF (SEQ ID NO: 10)
P57	CRSLFG (SEQ ID NO: 11)
P27	CRNLFG (SEQ ID NO: 12)
P21(N)	NCRRLFG (SEQ ID NO: 13)
P21(C)	KRRLIF (SEQ ID NO: 14)

In one embodiment of the invention multimeric repeats of Cy motifs controlled by an inducible system are used to provide multiple Cy inhibitory species in order to target CDK-cyclin activity. These Cy peptides are unlikely to be targeted by the 26 S proteasome, and thus afford a more stable means of triggering cell cycle inhibition and pseudo-senescence.

Antisense oligonucleotides also may be used to cause pseudo-senescence in a cell. Antisense methods have been used to block the cell cycle promoting activity of cyclins. Five stably transfected cell lines carrying a cyclin D1 antisense construct exhibited marked decrease of cell growth, in contrast to the original lines (A549 and NCI-H441) (Driscoll *et al.*, 1997). The expression of several cell cycle-regulating proteins, including cyclin A, the cyclin-dependent kinases (CDK) 2 and CDK4, in addition to cyclin D1 itself, were also markedly decreased. The expression of one CDK inhibitor, p21WAF1/CIP1, was increased in the A549-derived cell lines that were stably transfected with antisense cyclin D (Driscoll *et al.*, 1997). A specific target of cyclin D1 activity, the growth-suppressing product of the retinoblastoma gene, pRb exhibited decreased expression and a decreased level of phosphorylation in transfected cells. Decreased levels of pRb, due to a significant increase in its turnover rate, suggested that the stability of the protein may depend on phosphorylation by cyclin D1-dependent CDK activity. In

addition to the impact on pRb stability, decreased expression of cyclin D1 induced susceptibility to cell death after withdrawal of exogenous growth factors in the cell lines transfected with antisense constructs, a response that was not observed in the original cancer cell lines. Apparently, abrogation of cyclin D1 overexpression in cancer cells disrupts several key pathways that are required for uncontrolled cell growth and induces those that lead to cell death after growth factor deprivation (Driscoll *et al.*, 1997).

Conversely, using antisense oligonucleotides to block the actions of p27 leads to abrogation of quiescence (Rivard *et al.*, 1996), while antisense inhibition of p21 prevents EGF-mediated cell cycle arrest (Ohtsubo *et al.*, 1998).

One embodiment of this invention is to utilize antisense inhibitors of CDKs controlled by the inducible system to target CDK-cyclin activity via its activation rather than inhibition mechanism. Antisense technology can circumvent the processes of degradation of CKIs by the 26 S proteasome, and augments the RP shift via an alternative way of blocking the cell cycle and pseudo-senescence.

The present invention may also be used with yeast cells. Cellular and molecular studies of cyclins in invertebrate and vertebrate embryos have been preceded by genetic studies, particularly in ascomycete yeasts. In the fission yeast, the *cdc13* gene encodes a B-type cyclin that acts in cooperation with *cdc2* to regulate entry into mitosis (Booher & Beach, 1987; Booher & Beach, 1988; Booher *et al.*, 1989; Dunphy *et al.*, 1988; Gautier *et al.*, 1988; Hagan *et al.*, 1988; Solomon *et al.*, 1988).

Genetic studies in both the budding yeast and fission yeast have revealed that *cdc2* (or CDC28 in budding yeast) acts at two independent points in the cell cycle: mitosis and the so-called cell cycle "start" (Hartwell, 1971; Nurse & Bissett, 1981; Piggott *et al.*, 1982; Reed & Wittenberg, 1990).

In budding yeast, the start function of the CDC28 protein requires association of the catalytic subunit of the protein kinase with ancillary proteins that are structurally related to A and B-type cyclins. This third class of cyclin has been called the CLN class, and three genes comprising a partially redundant gene family have been described (Hadwiger *et al.*, 1989; Nash *et al.*, 1988; Richardson *et al.*,

1989). The CLN genes are essential for execution of start and in their absence, cells become arrested in the G I phase of the cell cycle. The CLN1 and CLN2 transcripts oscillate in abundance through the cell cycle, but the CLN3 transcript does not. In addition, the CLN2 protein has been shown to oscillate in parallel with its mRNA (Cross & Smith, 1988; Nash *et al.*, 1988; Richardson *et al.*, 1989; Wittenberg *et al.*, 1990).

Although the precise biochemical properties conferred on cdc2/CDC28 by association with different cyclins have not been fully elaborated, genetic studies of cyclin mutants clearly indicate that they confer "G1" and "G2" properties on the catalytic subunit (Booher & Beach, 1987; Nash *et al.*, 1988; Richardson *et al.*, 1989).

The genes that control the cell cycle are evolutionarily conserved (Nurse, 1990). Complementation of mutations in yeast cdc2 with human CDKs reactivated cell cycle control (Lee & Nurse, 1987). CKIs that inhibit yeast CDKs have been documented (Mendenhall, 1993; Schwob *et al.*, 1994). Since human CDKs are functional in yeast, the human CKIs may work as efficiently as the endogenous yeast forms. Therefore, the RP Shift vector containing human CKIs may be utilized in yeast. Blocking of cell division is to be assessed by measurements of cell number. Alternatively to the human CKIs, the endogenous yeast CKIs can be cloned into the RP shift vector.

The present invention also may be used with filamentous fungi. Most of the filamentous fungi important in biotechnology are members of the Deuteromycetes, including the *Aspergillus*, *Penicillium*, and *Trichoderma*. Basidiomycetes are also important in bioremediation. *Aspergillus* produces a number of secreted enzymes useful in the production of citric and gluconic acids that are both used as food additives. *Aspergillus* species express cyclins and CDKs with similar function to those found in yeast and human cells (Bussink & Osmani, 1998; Ye *et al.*, 1999). The RP shift system expressing human CKIs or Cy motifs may be used in filamentous fungi.

Furthermore, the present invention may be used with bacterial cells. Bacterial cell division follows a cell cycle pattern as well (Bramhill, 1997). Bacteria usually divide by building a central septum across the middle of the cell.

Apparently, the tubulin-like FtsZ protein plays a central role in cytokinesis as a major component of a contractile cytoskeleton. Assembly of FtsZ is a key point for regulation. The characterization of FtsZ homologues in Mycoplasmas, Archaea, and chloroplasts implies that the constriction mechanism is conserved, and that FtsZ can constrict in the absence of peptidoglycan synthesis (Bramhill, 1997). In most Eubacteria, the internal cytoskeleton must also regulate synthesis of septal peptidoglycan. The *E. coli* septum-specific penicillin-binding protein 3 (PBP3) forms a complex with other enzymes involved in murein metabolism, suggesting a centrally located transmembrane complex capable of splicing multiple new strands of peptidoglycan into the cell wall (Bramhill, 1997).

The cell cycle of *E. coli* does not have checkpoints, and several cycles may overlap (Donachie, 1993). It is the absence of such a checkpoint that results in the long filaments when division is blocked by mutation. *E. coli* cells divide when they achieve a certain cellular volume. Expression of a gene encoding a cell division inhibitor from the *sulA* locus is activated as a part of the bacterial SOS response following DNA damage (Bi & Lutkenhaus, 1993; Sonezaki *et al.*, 1995). Induction of the cell division inhibitor SulA, a component of the SOS response, or the inhibitor MinCD, a component of the min system, blocks formation of the FtsZ ring and leads to filamentation. Reversal of SulA inhibition by blocking protein synthesis in SulA-induced filaments leads to a resumption of FtsZ ring formation and division (Bi & Lutkenhaus, 1993; Sonezaki *et al.*, 1995). These results suggest that these inhibitors block cell division by preventing FtsZ localization into the central septum. In addition, analysis of min mutants demonstrated that FtsZ ring formation was also associated with minicell formation, indicating that all septation events in *E. coli* involved the FtsZ ring (Bi & Lutkenhaus, 1993). It is an embodiment of this invention that cloning active cell division inhibitors SulA and MinC into a bacterial compatible RP shift vector will afford a means of blocking the cell cycle in *E. coli*.

Expression Vectors

The present invention provides expression vectors allowing tight transcriptional regulation of an encoded polypeptide. Such vectors are useful in

methods of causing pseudo-senescence in a cell transfected, transformed, or infected with the vector.

In certain embodiments, cells are produced or maintained that contain one or more expression vectors encoding one or more proteins that contribute to the induction of a pseudo-senescent state in the cells. The vector may, for example, be in the form of a plasmid, cosmid, viral particle, or phage. The appropriate nucleic acid sequence may be inserted into the vector by a variety of procedures. In general, DNA is inserted into an appropriate restriction endonuclease site(s) using techniques known in the art. Vector components generally include, but are not limited to, one or more of a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence. Construction of suitable vectors containing one or more of these components employs standard ligation techniques that are known to the skilled artisan.

Expression vectors may contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2 micronx plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. However, in mammalian systems, it is preferred that the vector integrate into the genome thereby becoming dependent on the host for replication. Thus, in preferred embodiments, the vector is a retrovirus-based vector.

Expression vectors will typically contain a selection gene, also termed a selectable marker. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, *e.g.*, ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, *e.g.*, the gene encoding D-alanine racemase for *Bacilli*.

An example of suitable selectable markers for mammalian cells are those that enable the identification of cells comprising the expression vector, such as

DHFR or thymidine kinase. An appropriate host cell when wild-type DHFR is employed is the CHO cell line deficient in DHFR activity, prepared and propagated as described by Urlaub et al., Proc. Natl. Acad. Sci. USA, 77:4216 (1980). A suitable selection gene for use in yeast is the *trp1* gene present in the yeast plasmid YRp7 [Stinchcomb et al., Nature, 282:39 (1979); Kingsman et al., Gene, 7:141 (1979); Tschemper et al., Gene, 10:157 (1980)]. The *trp1* gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 [Jones, Genetics, 85:12 (1977)].

Expression vectors usually contain a promoter operably linked to the polypeptide-encoding nucleic acid sequence to direct mRNA synthesis. Promoters recognized by a variety of potential host cells are well known. Promoters suitable for use with prokaryotic hosts include the β -lactamase and lactose promoter systems [Chang et al., Nature, 275:615 (1978); Goeddel et al., Nature, 281:544 (1979)], alkaline phosphatase, a tryptophan (*trp*) promoter system [Goeddel, Nucleic Acids Res., 8:4057 (1980); EP 36,776], and hybrid promoters such as the *tac* promoter [deBoer et al., Proc. Natl. Acad. Sci. USA, 80:21-25 (1983)]. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the polypeptide encoding region.

Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase [Hitzeman et al., J. Biol. Chem., 255:2073 (1980)] or other glycolytic enzymes [Hess et al., J. Adv. Enzyme Reg., 7:149 (1968); Holland, Biochemistry, 17:4900 (1978)], such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase,

degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657.

5 Transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and Simian Virus 40 (SV40), from heterologous
10 mammalian promoters, *e.g.*, the actin promoter or an immunoglobulin promoter, and from heat-shock promoters, provided such promoters are compatible with the host cell systems.

 Transcription in higher eukaryotes may be increased by inserting an enhancer or repressor sequence into the vector. Enhancers and repressors are cis-
15 acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α -fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp
20 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

 In a preferred embodiment, an expression vector of the present invention contains a senescence-responsive element to increase the production of a recombinant protein. Additional amplification of the desired recombinant protein
25 is achieved by engineering a senescence-responsive element into the vector upstream of a CMV promoter. A senescence-responsive element has been defined at the -89 to -66 sequence (5'-AGGATGTTATAAAGCATGAGTCA-3' (SEQ ID NO:2)) of the human collagenase gene (Campisi & Testori, 1999). Triggering the senescence phenotype by expression of the CKIs activates senescence-specific
30 transcription factors, thereby accelerating transcription of the recombinant protein

of interest. In certain embodiments, the senescence-responsive element may be operably connected to a bicistronic construct comprising a combination of desired recombinant product and the IRES-driven cell cycle inhibitors separately or both transcribed from the regulated promoter. Such a dicistronic design provides simultaneous regulated expression of the target protein and the cell cycle regulator.

In certain embodiments, an expression vector of the present invention contains elements that allow tight regulation of gene expression. For example, the expression vector may contain one or more tetracycline repressor binding sites (tetracycline operators) in the promoter region of the vector. In a preferred embodiment, the vector comprises multiple tetracycline operators and a minimal promoter comprising a TATA sequence. Preferably, the tetracycline operators are arranged to provide tight regulation of the promoter. One such arrangement includes two phased tetracycline operators 21 basepairs downstream from the TATA sequence and two phased tetracycline operators 11 basepairs upstream from the TATA sequence.

When vectors comprising tetracycline repressor binding regions are used, it is necessary to deliver the tetracycline repressor into the cells chosen for biopharmaceutical production. The tetracycline repressor may be introduced into these producer cells via a retroviral transduction using IRES-containing single-transcript vector (Levenson *et al.*, 1998). After these producer cells are modified to express tetracycline repressor, the tetracycline-regulated construct containing the CKI is integrated into the genome of the producer cells by retroviral infection. Cells harboring the RP shift vector as stable transductants may be selected by resistance to the antibiotic G418. The expression of the delivered CKI or other cell-cycle inhibitor may then be induced by adding doxycycline (a stable derivative of tetracycline) into the media.

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally

3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding the polypeptide.

5 Bioreactors

 An important benefit of the present invention is the increase in protein yield from a cell culture. Many biotechnology products comprise a polypeptide isolated from a cell culture. Thus, many methods of isolating a desired protein from a cell culture are known in the art. The methods and compositions of the present invention may be utilized in conjunction with any of these methods to increase the yield of the protein.

 Bioreactors are essentially any device or means for culturing cells. Bioreactors and methods of isolating proteins therefrom are well known in the art. Examples of bioreactors and their methods of use are described in U.S. Patent Nos. 6,214,221; 6,100,061; 5,998,184; and 5,571,720. Bioreactors of the present invention include those that are stop-flow and flow-through.

EXAMPLES

 The following examples are intended to illustrate the present invention without limitation.

Example 1

 This example describes the construction of a tetracycline-regulated retroviral vector (LNtCtX with PolyA). This expression vector can be (1) tightly regulated, to allow robust cell growth when in the OFF position; (2) is highly inducible by inexpensive and FDA-approved ligand; (3) is very promiscuous to allow efficient incorporation and subsequent expression in a wide variety of cells. The LNtCtX with PolyA vector was built from the mouse leukemia retroviral vector LNCX (W. S. Pear, G. P. Nolan, M. L. Scott, D. Baltimore, *Proc Natl Acad Sci U S A* **90**, 8392-6 (1993) with several important modifications. Its final form contains the viral long terminal repeat (LTR), a selection marker, and a multiple cloning site flanked by a polyadenylation signal and a regulated promoter (see

FIG. 1). The sequence of the vector is provided in Table 2. A Mo-MSV LTR is encoded by basepairs 145-795 and basepairs 4089-4793. The retroviral packaging sequence is encoded within basepairs 796-1132. The neomycin resistance gene is encoded within basepairs 1194-2450. The bovine growth hormone poplyadenylation signal is encoded within basepairs 3074-2843. Basepairs 3217-3176 and 3282-3235 each contain two tetracycline operators. Basepairs 3889-3235 encode a CMV promoter. Whereas basepairs 4794-7086 encode pBR322 DNA. A diagram of the LNTcX with PolyA vector is provided by FIG. 2.

Table 2
Sequence of the RP shift vector (SEQ ID NO:1)

gaattcatac	cagatcaccg	aaaactgtcc	tccaaatgtg	tccccctcac	50		
actcccaa	at	tgcg	gggctt	ctgcctctta	gaccactcta	ccctattccc	100
cacactcacc	ggagccaaag	ccgcggccct	tccgtttctt	tgcttttgaa	150		
agaccccacc	cgtaggtggc	aagctagctt	aagtaacgcc	actttgcaag	200		
gcatggaaaa	atacataact	gagaatagaa	aagttcagat	caagggtcagg	250		
aacaaagaaa	cagctgaata	ccaaacagga	tatctgtggt	aagcgggttc	300		
tgccccggct	cagggccaag	aacagatgag	acagctgagt	gatggggcaa	350		
acaggatatc	tgtggtaagc	agttcctgcc	ccggctcggg	gccaagaaca	400		
gatgggtcccc	agatgcggtc	cagccctcag	cagtttctag	tgaatcatca	450		
gatgtttcca	gggtgcecca	aggacctgaa	aatgaccctg	taccttattt	500		
gaactaacca	atcagttcgc	ttctcgcttc	tgttcgcgcg	cttcgcgtct	550		
ccgagctcaa	taaaagagcc	cacaaccct	cactcggcgc	gccagtcttc	600		
cgatagactg	cgtcgcccgg	gtacccgat	tcccaataaa	gcctcttgct	650		
gtttgcatcc	gaatcgtggt	ctcgtgttc	cttgggaggg	tctcctctga	700		
gtgattgact	accacgacg	ggggtctttc	atttgggggc	tcgtccggga	750		
tttgagagacc	cctgcccagg	gaccaccgac	ccaccaccgg	gaggttaagct	800		

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ggccagcaac ttatctgtgt ctgtccgatt gtctagtgtc tatgtttgat      850
gttatgcgcc tgcgtctgta ctagttagct aactagctct gtatctggcg      900
gacccgtggt ggaactgacg agttctgaac acccggccgc aaccctggga      950
gacgtcccag ggactttggg ggccgttttt gtggcccgcac ctgaggaagg     1000
gagtcgatgt ggaatccgac cccgtcagga tatgtggttc tggtaggaga     1050
cgagaaccta aaacagttcc cgcctccgtc tgaatttttg ctttcggttt     1100
ggaaccgaag ccgcgcgtct tgtctgctgc agcgtgcag catcgttctg     1150
tgttgtctct gtctgactgt gtttctgtat ttgtctgaaa attagggcca     1200
gactgttacc actcccttaa gtttgacctt aggtcactgg aaagatgtcg     1250
agcggatcgc tcacaaccag tcggtagatg tcaagaagag acgttggggt     1300
acettctgct ctgcagaatg gccaaccttt aacgtcggat ggccgcgaga     1350
cggcaccttt aaccgagacc tcatcaccca ggttaagatc aaggtctttt     1400
cacctggccc gcatggacac ccagaccagg tcccctacat cgtgacctgg     1450
gaagccttgg cttttgacce ccctccctgg gtcaagccct ttgtacaccc     1500
taagcctccg cctcctcttc ctccatccgc cccgtctctc ccccttgaac     1550
ctcctcgttc gaccccgctt cgatcctccc tttatccagc cctcactcct     1600
tctctaggcg ccggaattcc gatctgatca agagacagga tgaggatcgt     1650
ttcgcatgat tgaacaagat ggattgcacg caggttctcc ggccgcttgg     1700
gtggagaggg tattcggcta tgactgggca caacagacaa tcggctgctc     1750
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The Moloney strain murine leukemia virus vector LNCX contains a regulated promoter and polyadenylation site are positioned in reverse orientation to the LTR to prevent read-through from the LTR, thereby eliminating LTR-initiated expression leakage. At the same time, reverse orientation of polyadenylation signal does not interfere with genomic RNA transcription in packaging cells.

LNCX was modified according to Kistner to contain an array of seven cognate tetracycline operator sequences linked to a minimal CMV promoter which is itself inactive (A. Kistner, et al., *Proc Natl Acad Sci U S A* **93**, 10933-8. (1996). This arrangement was additionally modified in the LNCtX with PolyA by the insertion of two phased tetracycline operators 21 bp downstream from the TATA site and two phased tetracycline operators 11 bp upstream of the TATA sequence within the CMV promoter. This configuration positions a tight protein clamp of two dimerized TetR elements both in front of the TF-IIID contact site and also exactly at the site of initiation of transcription. Moreover, binding of dimerized tetracycline repressors induces a significant kink in the double helix, further reducing the probability of fortuitous transcription.

cDNAs for the CKIs p16, p21, p27, and p57, in sense orientation, are individually cloned into the multiple cloning site (MCS) to be expressed from the regulated promoter. These vectors are referred to herein as RP shift vectors.

Example 2

This example describes the production of infectious viral particles using a expression vector of the present invention and the subsequent infection of target cells. This delivery system employs a pantropic system to deliver the DNA to the cells. VSV-G, an envelope glycoprotein, is used to mediate viral entry into cells through lipid binding and plasma membrane fusion (J. C. Burns, T. Friedmann, W. Driever, M. Burrascano, J. K. Yee, *Proc Natl Acad Sci U S A* **90**, 8033-7 (1993); J. K. Yee, et al., *Proc Natl Acad Sci U S A* **91**, 9564-8 (1994); and N. Emi, T. Friedmann, J. K. Yee, *J Virol* **65**, 1202-7 (1991)). Because this system does not depend on specific cell surface receptors, the pantropic system allows transduction of any mitotically active cells. Infectious pantropic retroviral particles carrying the gene of interest were transfected into GP2-293 cells using standard Ca-phosphate (W. S. Pear, G. P. Nolan, M. L. Scott, D. Baltimore, *Proc Natl Acad Sci U S A* **90**, 8392-6 (1993) technique. Twenty-four hours after transfection culture medium with infectious virions was collected, filtered through 0.45 μ m filter to remove stray packaging cells, supplemented with PolybreneTM (4 μ g/ml) and added to the target cells. Twenty-four hours later cells were trypsinized and replated: two 60-mm plates will be seeded with 200 cells each, while the rest of the cells is plated into 150-mm plates at a density of 10^6 cells per plate. All cells in 150-mm plates and one of the 60-mm plates were selected with G418 (0.7 mg/ml for 10 days) to eliminate uninfected cells. The second 60-mm plate is left without antibiotic. An infection rate of 55% was determined by colony formation by dividing the number of colonies in G418-treated 60-mm plate by the number of colonies in its untreated companion.

Since retroviral rearrangements are expected in 10-15% of infected cells (V. V. Levenson, E. D. Transue, I. B. Roninson, *Hum Gene Ther* **9**, 1233-6 (1998) it was desirable to isolate 10-20 individual colonies from the infected and G418-selected 150-mm plate.

Target cell lines must be able to encounter and receive viral particles produced by packaging cell lines. In the case of human cells, VSV-G viral coat

proteins are used so that fusion of the viral particles to plasma membrane affords infection of target cells. In the case of hybridoma cells, which are formed by fusing human myeloma cells with mouse splenocytes so that they express ecotropic receptor, mouse derived retroviral vectors may be used.

5

Example 3

In this Example, the present invention is used to increase the production of the secreted enzyme plasminogen activator inhibitor type 1 (PAI-1). HT1080 E-14 cells actively produce PAI-1 (S. H. Kang, et al., *Int J Cancer* **77**, 620-5 (1998)). HT1080 E-14 cells expressing tetracycline repressor with a nuclear localization
10 signal (A. Kistner, et al., *Proc Natl Acad Sci U S A* **93**, 10933-8. (1996)) were prepared via a retroviral transduction using an IRES-containing single-transcript vector (V. V. Levenson, E. D. Transue, I. B. Roninson, *Hum Gene Ther* **9**, 1233-6 (1998)). After these producer cells were modified to express tetracycline
15 repressor, a tetracycline-regulated RP shift construct containing a CKI (p16) was integrated into the genome of the producer cells by retroviral infection. Cells harboring the RP shift vector as stable transductants were selected by resistance to the antibiotic G418.

The CKI was induced by addition of 2 μ M doxycyclin. Cell proliferation, as monitored by uptake of methylene blue dye, was immediately blocked. Cells
20 exposed to doxycyclin develop a large, flattened appearance associated with the senescence phenotype (Campisi, 2000). Furthermore, the onset of pseudo-senescence was observed by staining cells for senescence associated- β -galactosidase (G. P. Dimri, et al., *Proc Natl Acad Sci U S A* **92**, 9363-7 (1995)).

The amount of plasminogen activator inhibitor-1, a protein normally
25 secreted by HT1080 cells, was monitored by ELISA assays using anti-PAI-1 alkaline phosphatase conjugate (S. M. Wileman, et al., *Br J Ophthalmol* **84**, 417-22 (2000)). As FIG. 3 indicates, secreted protein levels were increased by as much as 30-fold.

Example 4

This example demonstrates that the present invention is useful for increasing the production of monoclonal antibodies from hybridoma cell lines. Since hybridoma cells contain ecotropic receptor, they should be infected by RP Shift retrovirus without use of VSV-G. To demonstrate the infectability of hybridoma cells, Enhanced Green Fluorescent Protein (Clontech Laboratories) was cloned into the RP Shift vector. This retroviral vector (10 μ g) was transfected into 5 x10⁶ BOSC packaging cells and media containing viral particles was added to 500,000 hybridoma cells twice over 48 h. The percent infected cells enhanced green fluorescent protein was monitored by flow cytometry 48 h after addition of 2 μ M doxycyclin.

The expression of CKIs p21, p57, and p16 in these cells were also assessed by RT-PCR. Cells harboring CKI constructs were selected using appropriate G418 (1 mg/ml) for the resistance genes engineered into the retroviral construct. Expression of the CKIs prevents cell cycle progression and induces a pseudo-senescent state blocking further cell division and expanding the protein synthesis capacity of the cells. The effects of RP Shift on cell cycle progression in hybridoma cells was assessed by monitoring DNA content of the cells. S-phase, the segment of the cell cycle when DNA is duplicated, was suppressed in cells undergoing RP Shift. This result indicates that the hybridoma cells had stopped dividing. Cells in the pseudo-senescent state were tested for resistance to apoptosis by assessing cell viability and cell number after 3 weeks in culture. Cell number varied little over this time period, indicating that the cells are stable in long term culture.

The production of monoclonal antibody was monitored by ELISA using anti-IgG alkaline phosphatase conjugate. The population of hybridoma cells undergoing RP Shift increased production of monoclonal antibody by nearly 8-fold after one week in culture. These examples demonstrate that RP Shift can be used to block cell division of mammalian cells and a concomitant enhanced production of native protein from these cells.